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ORIGINAL PAPER

Regioselective galloylation of methyl β-D-glucopyranoside by a lipase

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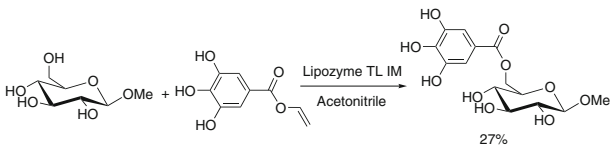
Abstract Chromogenic substrate 4-nitrophenyl gallate was prepared in four steps and used for selection of hydrolases specific to gallic ester hydrolysis from among 22 commercial lipases, proteases, and crude glycanase cocktails. Enzymes displaying galloyl esterase activity were tested in regioselective galloylation of methyl β-D-glucopyranoside with vinyl gallate. Lipozyme TL IM in acetonitrile was found to afford the highest conversion (37 %). The reaction proceeded with strict regioselectivity toward the primary hydroxyl of the glucopyranoside ring, giving in preparative scale 27 % of purified methyl 6-*O*-galloyl-β-D-glucopyranoside as a sole product.

Graphical abstract



Keywords Acylation · Enzymes · Glycosides · Regioselectivity

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Introduction

The structural motif of 6-*O*-galloyl-β-D-glucopyranos-1-*O*-yl (Fig. 1) is an abundant constitutive fragment of bioactive natural substances. Many of these compounds were isolated from leaves, stems, roots, or seeds of medicinal plants and possess remarkable biological activities when bearing, for example, methyl [1], another galloyl [2, 3], *p*-hydroxyphenyl [4], *p*-hydroxy-3,5-dimethoxyphenyl [5, 6], or 5-hydroxyeugenyl [7, 8] moiety as the aglycone.

Chemical synthesis of such types of molecules is typically a multistep process which involves several selective protection and deprotection steps as described for example in the preparation of 1,6-di-*O*-galloyl-β-D-glucopyranose [9]. On the other hand, direct enzymatic regioselective galloylation of appropriate glucoside may provide a simple, more straightforward alternative to standard processes. There are however no literary data providing effective conditions for enzyme-catalyzed galloylation of either sugars or generally polyhydroxylated compounds.

The majority of published reports on enzymatic galloylations concerns preparation of alkyl gallates [10, 11], with focus on synthesis of industrially exploited antioxidant—propyl gallate—by microbial tannases [12]. Two main approaches of preparation of propyl gallate are based either on direct esterification of gallic acid (**1**) by *n*-propanol under catalysis of tannases and lipases in organic media [10, 11, 13–15] or by exploitation of tannic acid as donor for gallate transfer catalyzed by esterase and depsidase activity of tannases [16–21]. Transesterifications in optimized conditions may provide chemical yields of propyl gallate as high as 90 % [17, 19, 20]. This method is, however, not suitable for preparation of galloyl glycosides due to complicated separation of chemically similar products from the reaction mixture. Moreover, galloylation of

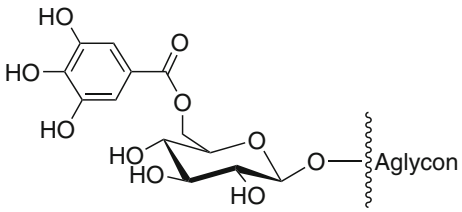

Fig. 1 6-*O*-galloyl-β-D-glucopyranos-1-*O*-yl structural motif

structurally more sophisticated acceptors than *n*-propanol is less efficient as proven in direct acylation of catechins with gallic acid catalyzed by immobilized tannase with chemical yields not exceeding 6 % [22]. To our best knowledge, the only successful enzymatic galloylation of sugars was reported in our recent work [23]. Within our investigation of substrate specificity and regioselectivity of Lipolase 100T on wide scale of aromatic donors we carried out galloylation of methyl α-D-glucopyranoside, achieving only 12 % yield of 6-*O*-galloylated product.

The purpose of this study was therefore to find either an enzyme displaying galloyl esterase (tannase) activity or a lipase with wider substrate specificity and therefore able to catalyze more effective galloylation of model glycoside as a transfer of gallate from its activated donor. At the same time, the selected enzyme should be active in polar organic solvents, which are necessary for dissolving the glycosidic acceptor.

Results and discussion

Synthesis of substrates

To select the appropriate enzyme and conditions for effective acylation of the model saccharide with gallic acid (**1**), we have prepared two types of gallic esters—4-nitrophenyl gallate (4-nitrophenyl 3,4,5-trihydroxybenzoate, **2**) for galloyl esterase assays and vinyl gallate (vinyl 3,4,5-trihydroxybenzoate, **3**) as the activated galloyl donor for preparative syntheses. Preparation of the substrate **3** was executed according to the method published by our group elsewhere [23]. The first synthesis of **2** as a substrate for spectrophotometric assay of tannases was realized in 1970 by Haslam and Tanner [24]. 4-Nitrophenyl protocatechuate—another similar chromogenic substrate for tannase assays was synthetized in 2000 [25]. Both syntheses were based on protection of phenolic *ortho*-hydroxyls by dichlorodiphenylmethane. Our simplified procedure to prepare **2** includes four easily consecutive steps (Scheme 1): 3,4,5-triacetoxybenzoyl chloride (**4**) was prepared from fully acetylated **1** [26] and the 4-nitrophenyl 3,4,5-triacetoxybenzoate obtained by base catalyzed esterification was directly deacetylated in acidic conditions.

Screening of galloyl esterases

The modified photometric method of Haslam and Tanner [24] was used for routine screening of galloyl esterase activity among a scale of commercial lipases, proteases, and crude glycanases either in solid or in liquid form. Liquid preparations, if positive for galloyl esterase, were planned to be ultrafiltered and lyophilized in view of their use as solid catalysts in organic solvents. Measurable activities were found in three lipase preparations and one glycanase, all from *Thermomyces lanuginosus* (orig. *Humicola lanuginosa*) (Table 1). We expected galloyl esterase activity also in lipase B from *Candida antarctica* due to its wide substrate specificity, including phenolic acids [27, 28]. The physical properties of its immobilized preparation Novozyme 435, however, did not allow us to measure the activity by photometric method—the non-soluble enzyme particles were freely flowing on the surface of the buffer solution or adhered on the walls of the reaction vessel above the liquid.

These five enzymes were tested in acylation of methyl β-D-glucopyranoside (**5**) with **3** (Scheme 2). It is worth to point that the conversions after 15 days (Table 2) did not correspond to galloyl esterase activities displayed by the respective enzymes as summarized in Table 1. Only two enzymes, namely Lipozyme TL IM and Lipex 100T, were moderately effective in the reaction, reaching the respective conversions 29.8 and 12.1 %. Lipozyme TL IM was therefore our choice of biocatalyst for further selection of the reaction conditions.

Choice of solvent and excess of galloyl donor

Enzymatic acylation of saccharides with (poly)phenolic acids obviously encounters the problem of mutual incompatibility of the catalyst, reactants and the reaction environment. Choice of reaction solvent must respect hydrophilic character and solubility of reactants, while lipase-catalyzed esterifications and transesterifications better proceed in hydrophobic environment with low content of water. We screened four polar or moderately polar organic solvents to find the most suitable one for the studied transgalloylation. Among them, acetone and acetonitrile (Fig. 2) allowed us to achieve the highest levels of galloylated methyl β-D-glucopyranoside (methyl 6-*O*-(3,4,5-trihydroxybenzoyl)-β-D-glucopyranoside, **6**). Acetonitrile was therefore used as reaction solvent in further experiments.

Increased ratio of **3** as a galloyl donor to the acceptor **5** negatively influenced the process of its galloylation (Fig. 3). Despite of higher reaction rates, the final product concentrations decreased and the best results were achieved with 1.5 equivalents of vinyl gallate. The level of galloylation finished at 37 % after 48 days while reaction with 3 or 5

Scheme 1

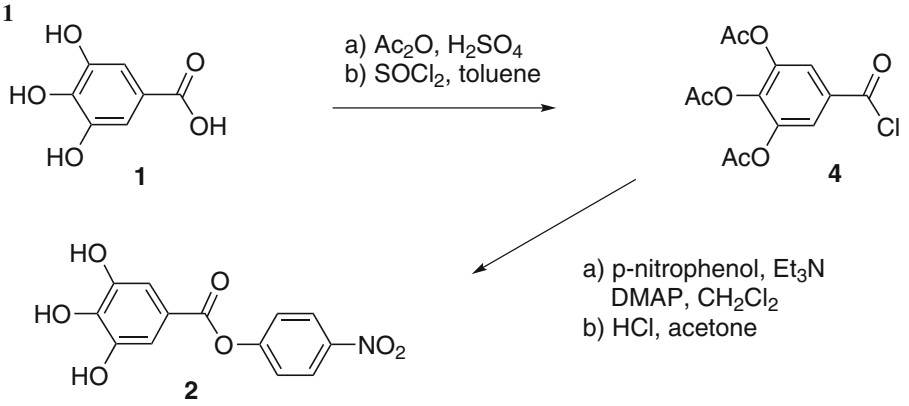

Table 1 Galloyl esterase activity of selected hydrolases

Enzyme (type)	Origin	Activity[a]
Lipex 100T (lipase)	*Thermomyces lanuginosus*	0.05[b]
Pentopan 500BG (xylanase)	*Thermomyces lanuginosus*	9.6[b]
Lipozyme TL IM (lipase)	*Thermomyces lanuginosus*	0.05[b]
Novozym 435 (lipase)	*Candida antarctica*	n.d.
Lipolase 100T (lipase)	*Thermomyces lanuginosus*	0.17[b]
Lipolyve AN (lipase)	*Aspergillus niger*	0[b]
PPL (lipase)	pig pancreas	0[b]
Lipase PS (lipase)	*Burkholderia cepacia*	0[b]
Lipase AK (lipase)	*Pseudomonas fluorescens*	0[b]
α-chymotrypsin (protease)	bovine pancreas	0[b]
Protease NL (protease)	*Bacillus subtilis*	0[c]
Liquanase Ultra 2,5L (protease)	*Bacillus subtilis*	0[c]
Corolase L10 (protease)	*Carica papaya*	0[c]
Lallzyme Beta (pectinase)	*Aspergillus niger*	0[b]
Lallzyme Cuvee Blanc (pectinase)	*Aspergillus niger*	0[b]
Cytolase M102 (pectinase)	*Aspergillus niger*	0[b]
Rapidase Expression (pectinase)	*Aspergillus niger*	0[b]
Ultrazym 100 (pectinase)	*Aspergillus niger*	0[b]
Novozym 188 (cellobiase)	*Aspergillus niger*	0[c]
Dextrozyme GA 1,5X (glucoamylase)	*Aspergillus niger*	0[c]
Celluclast 1.5 L (cellulase)	*Trichoderma reesei*	0[c]
Carezyme 4500 L (cellulase)	*Aspergillus* sp.	0[c]

[a] One unit (IU) corresponds to amount of enzyme forming 1 μmol of p-nitrophenol per 1 min

[b] IU/g

[c] IU/cm^3

equivalents of vinyl gallate reached plateau of 26–27 and 23 % conversion after 22 and 3 days, respectively.

Preparative reaction, isolation and structure of the product

Preparative galloylation of methyl β-D-glucopyranoside catalyzed with Lipozyme TL IM was carried out in acetonitrile with 1.5 equiv. of vinyl gallate and the reaction was shaken at 40 °C for 34 days. The reaction mixture provided the product in amount corresponding to 27 % chemical yield. Structural analysis of the product by nuclear magnetic resonance had proven that the reaction proceeds strictly regioselectively toward the primary hydroxyl of the acceptor. No formation of digalloyl derivatives of **5** was observed. Such regioselectivity in

Scheme 2

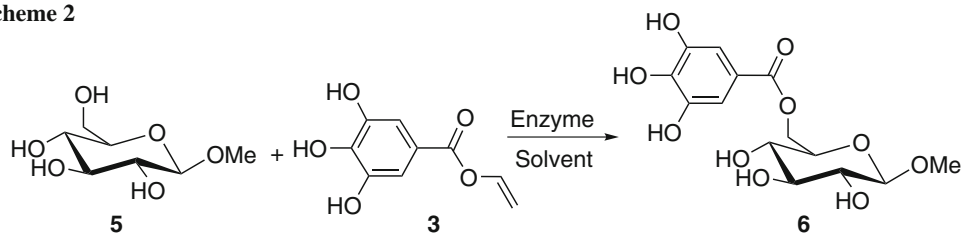

Table 2 Galloylation of **5** catalyzed by hydrolases in acetonitrile

Enzyme	Reaction time/h	Conversion/ %
Pentopan 500BG	360	2.1
Lipolase 100T	360	3.3
Novozym 435	360	3.7
Lipex 100T	360	12.1
Lipozyme TL IM	360	29.8

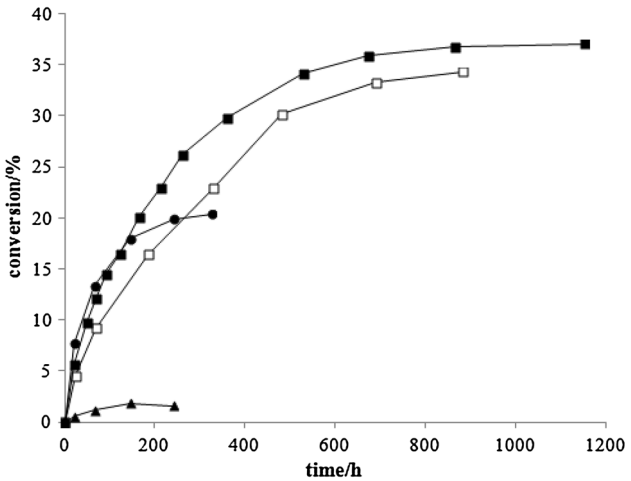

Fig. 2 Effect of reaction media on galloylation of **5** with 1.5 equiv. of vinyl gallate (**3**) catalyzed by Lipozyme TL IM. Reactions conducted in acetone (*empty square*), *tert*-butanol (*filled circle*), isobutyl methyl ketone (*filled triangle*) and acetonitrile (*filled square*)

acylation of primary hydroxyl of acceptor is consistent with our experience with another commercial lipase from *Thermomyces lanuginosus* (Lipolase 100T). Acylation of model glycoside with aromatic vinyl esters bearing free phenolic hydroxyls on aromatic ring (including vinyl gallate) proceeded strictly to the primary position in low yields. On the other hand, the regioselectivity changed if the aromatic ring was methoxylated or the hydroxyls were missing. The reactions proceeded faster, with higher yields and mixtures of monoesters (at the primary hydroxyl) and corresponding 2,6-diacylated glycoside were formed [23].

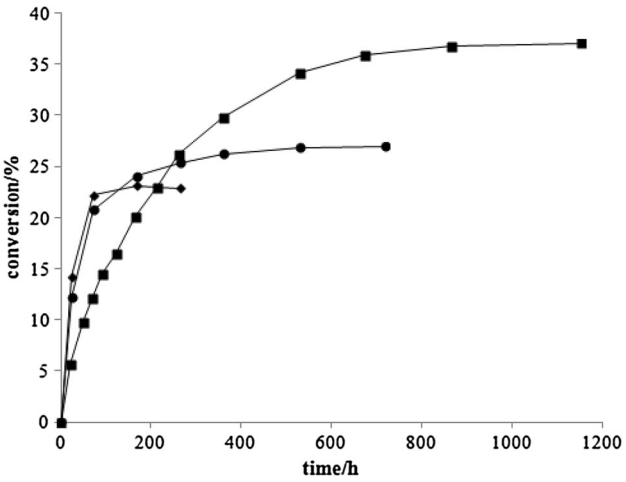

Fig. 3 Effect of excess of vinyl gallate on galloylation of **5** by Lipozyme TL IM. Reactions conducted in acetonitrile (*filled square* 1.5 equiv., *filled circle* 3 equiv., *filled diamond* 5 equiv.)

Conclusion

Chromogenic substrate 4-nitrophenyl gallate for assay of enzymes hydrolysing gallic ester bond was prepared by new synthetic way in four steps. The photometric assay identified three lipases and one xylanase (all from *Thermomyces lanuginosus*) comprising gallic ester hydrolase activity. These enzymes, together with lipase B from *Candida antarctica* were tested in galloylations of model glycoside with vinyl gallate as galloyl donor. The galloylation of methyl β-D-glucopyranoside was achieved in moderate yield through transesterification catalyzed by Lipozyme TL IM in acetonitrile. The regioselectivity of the reaction proceeded exclusively at the primary hydroxyl of the glucopyranoside. According to our knowledge, this is the most effective enzymatic galloylation of saccharide reported so far. The product of the reaction is a natural substance occurring in *Sanguisorba officinalis* L. [1]. The reaction presented in this work opens also access to other biologically active galloylated glycosides of plant origin.

Experimental

Enzyme preparations from Novozymes—Lipex 100T, Pentopan 500 BG, Lipozyme TL IM, Novozym 435, Lipolase 100T, Liquanase Ultra 2,5L, Corolase L10, Novozym 188, Dextrozyme GA 1,5X, Celluclast 1,5L and Carezyme 4500L—were gifts from MSc. Marián Illáš (Biotech s.r.o., Slovakia), Lipase AK, Lipase PS and Protease NL were purchased from Amano Enzyme (USA). Lallzyme BETA and Lallzyme Cuvée Blanc were purchased from Lallemand (Canada). Lipolyve AN was gift from Lyven (France). α-Chymotrypsin and pig pancreatic lipase were from Sigma (St. Louis, MO, USA). Ultrazym 100 was from Ciba Geigy. Rapidase Expression was gift from O.K.SERVIS BioPro, s.r.o. (Czech Republic, local supplier of DSM), Cytolase M102 from DSM was ultrafiltered and lyophilized before use. Gallic acid (**1**) and molecular sieves were purchased from Sigma Chemical (St. Louis, MO, USA), and methyl β-D-glucopyranoside hemihydrate (**2**) was purchased from Acrōs Organics (New Jersey, USA). The methyl 6-*O*-galloyl-β-D-glucopyranoside (**6**) used as the standard for HPLC was prepared by non-optimized enzymatic reaction. Vinyl gallate **3** was prepared according to the method described by Mastihubová et al. [23]. Organic solvents for synthesis were dried and distilled before use. All other chemicals were of analytical or HPLC grade.

TLC alumina plates Silica gel 60 F254 from Merck KGaA (Darmstadt, Germany) were used for TLC analysis. The plates were eluted by ethyl acetate/methanol (9:1, v/v) and the spots were detected by charring the plates with 5 % (v/v) ethanolic H_2SO_4 and heating at ca. 200 °C. High-performance liquid chromatography was performed on an Agilent 1200 Series apparatus (Agilent Technologies, Inc., Santa Clara, CA, USA) with MZ Aqua Perfect C18 5-μm reverse-phase column (150 × 4.0 mm) from MZ-Analysentechnik (Mainz, Germany). Spectrophotometric assays were performed on a UV–visible (UV–Vis) spectrophotometer (UV-1800, Shimadzu). Optical rotations were measured with a digital polarimeter Jasco P-2000 at 20 °C. ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (101 MHz) were recorded with a Varian 400 MHz spectrometer. High resolution mass spectra (HR-MS) were obtained with the Orbitrap Velos PRO spectrometer from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

Quantitative analysis of reaction mixtures by HPLC

The column was equilibrated/eluted with a mixture of water/acetic acid/1-butanol (500:1:2.5, v/v/v) with a flow rate of 1 cm^3/min, and a gradient of methanol (ramped up to 50 % between 12 and 22 min and then back to 0 % in 8 min) was applied. Column thermostat was set up to 30 °C and RI detector to 35 °C. Individual components were detected at 275 nm and the amount of galloylated product was calculated from the calibration curve with linear relation in the interval 0.297–2.97 mM.

Synthesis of 4-nitrophenyl gallate (**2**)

To 3.14 g 3,4,5-tri-*O*-acetoxybenzoyl chloride **4** [26] (10 mmol) and 1.53 g *p*-nitrophenol (11 mmol) dissolved in 50 cm^3 dichloromethane, 1.24 cm^3 triethylamine (8.5 mmol) and 0.32 g DMAP (2.5 mmol) were added at 0 °C. The reaction mixture was then stirred under argon atmosphere at laboratory temperature for 1 h. The reaction mixture was then diluted with 50 cm^3 dichloromethane, washed with 40 cm^3 1 % HCl, 50 cm^3 brine, sat. $NaHCO_3$ (2 × 50 cm^3) and brine (2 × 50 cm^3), dried over Na_2SO_4, and concentrated under reduced pressure. Crude honey-like material (4.4 g) was dissolved in 60 cm^3 acetone and 30 cm^3 6 M HCl was added. The reaction mixture was stirred at laboratory temperature for 48 h, then neutralized by solid Na_2CO_3 and acetone was eliminated under reduced pressure. The product was extracted from water phase by EtOAc (3 × 50 cm^3), organic phase was washed by water (2 × 20 cm^3), dried over Na_2SO_4 and concentrated in vacuo to obtain a crude residue. This residue was purified by flash chromatography (toluene:EtOAc 3:1). The fractions containing **5** were partly concentrated under reduced pressure until the moment when the product **5** started to precipitate. The mixture was left in the cold for several hours, solids were filtered, washed by cold toluene, and dried. Yield 2.11 g (72 %, two steps); pale yellow crystals; m.p.: 196–198 °C ($CH_3OH/CHCl_3$) (Ref. [24] 197–198 °C); R_f (toluene/EtOAc, 1:1, v/v) = 0.34; ^{1}H NMR [400 MHz, $(CD_3)_2CO$]: δ = 8.39 (br s, 3H, OH), 8.35 (d, 2H, J = 9.2 Hz, H-Ar), 7.56 (d, 2H, J = 9.2 Hz, H-Ar), 7.29 (s, 2H, H-Ar) ppm; ^{13}C NMR (101 MHz, $(CD_3)_2CO$): δ = 164.7 (COO), 157.2 (C-Ar), 146.3 (2 × C–OH), 146.2 (C-Ar), 140.0 (C–OH), 125.9 (2 × CH-Ar), 123.9 (2 × CH-Ar), 120.0 (C-Ar), 110.7 (2 × CH-Ar) ppm.

Assay of galloyl esterase

Galloyl esterase activity was determined according to the modified spectrophotometric assay described in the report of Haslam [24]. The analytical reaction was performed in 10 cm^3 DURAN flasks with a sealing lid. Portion of 5 cm^3 of freshly prepared 1 mM solution of 4-nitrophenyl gallate was transferred into each flask and the reaction was placed on a rotating shaker set up to 250 rpm in thermostat at constant temperature 40 °C. The reaction was initiated by adding enzyme preparation (100 mg) except control

sample (blank). In 10 min intervals, 1 cm^3 aliquots of the reaction mixture were withdrawn and passed through a 0.22-μm nylon syringe filter. The change of absorbance was read at 405 nm in 10-mm path length polystyrene cuvettes. After reading the absorbance, the solutions were returned back to reaction flasks.

Enzymatic transgalloylations

A typical reaction for screening of reaction conditions was performed in 10 cm^3 DURAN flasks with a sealing lid on a vibrating shaker set up to 300 rpm in thermostat at constant temperature 40 °C. The reaction mixture comprised 0.04 M methyl β-D-glucopyranoside **5**, 0.06 M vinyl gallate **3**, 0.4 g of activated molecular sieves 4Å, and 0.4 g of enzyme preparation (except blank) in 5 cm^3 of acetonitrile. To monitor the reaction course, 50-mm^3 aliquots of the reaction mixture were withdrawn at predefined time intervals and analyzed by HPLC.

Methyl 6-O-galloyl-β-D-glucopyranoside (**6**)
The preparative reaction was scaled up 10 times. The reaction mixture comprised 406 mg of methyl β-D-glucopyranoside **5** (2 mmol), 588 mg of vinyl gallate **3** (3 mmol), 4 g of activated molecular sieves 4 Å, and 4 g of Lipozyme TL IM in 50 cm^3 of acetonitrile. The reaction mixture was stopped after 34 days by filtration, the filter cake was washed with acetonitrile and washings were mixed with the filtrate. Activated charcoal was added and the suspension was briefly heated. The mixture was filtered again and solvents from the filtrate were removed by evaporation. The residue was purified by chromatography on the column of silica-gel eluted with EtOAc. Fractions with pure product were collected and the solvent was removed by evaporation. The honey-like residue was precipitated from toluene and separated by filtration to obtain 0.184 g (27 %) of pale yellow amorphous solid. R_f (EtOAc/methanol, 4:1, v/v) = 0.32; $[\alpha]_D^{20} = -15.2°$ ($c = 1.0$, CH_3OH), [Ref. [1] $[\alpha]_D^{22} = -18.6°$ ($c = 1.3$, H_2O)]; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.09$ (s, 2H, H-Ar), 4.89 (bs, 3H, 3 × OH), 4.55 (dd, 1H, $J = 11.9$, 2.1 Hz, H-6a), 4.39 (dd, 1H, $J = 11.9$, 5.6 Hz, H-6b), 4.22 (d, 1H, $J = 7.8$ Hz, H-1), 3.56 (ddq, 1H, $J = 7.2$, 3.7, 1.9 Hz, H-5), 3.50 (s, 3H, OCH_3), 3.46–3.37 (m, 2H, H-3, H-4), 3.21 (dd, 1H, $J = 9.2$, 7.9 Hz, H-2) ppm; ^{13}C NMR (101 MHz, CD_3OD): $\delta = 168.3$ (COO), 146.5 (2 × C–OH), 139.8 (C–OH), 121.4 (C-Ar), 110.1 (2 × CH-Ar), 105.4 (C-1), 77.9, 71.6 (C-3, C-4), 75.4 (C-5), 75.0 (C-2), 64.7 (C-6), 57.3 (OCH_3) ppm; HRMS (ESI): *m/z* calcd for $C_{14}H_{18}O_{10}Na$ ($[M + Na]^+$) 369.07977, found 369.07911.

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